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Translation No. 1726

DECEMBER 1965

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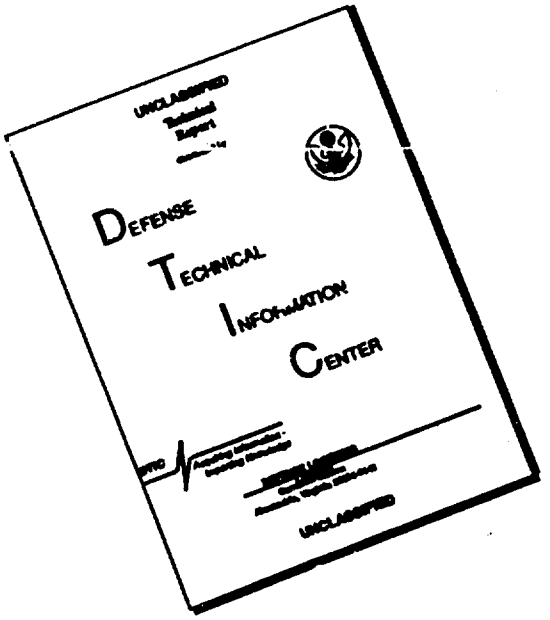

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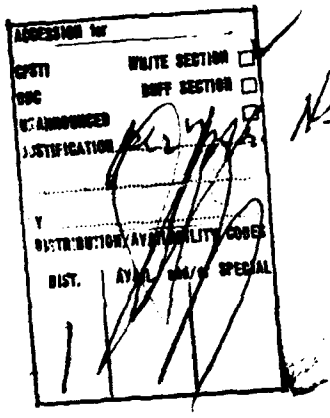

E. COLI VARIABILITY IN WATER UNDER THE EFFECT OF OZONE

Following is the translation of an article by K. K. Vrochinskiy, Kiev Oblast Sanitary-Epidemiological Station and the Communal Hygiene Faculty of the Kiev Medical Institute, published in the Russian-language periodical Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology and Immunobiology), No 6, 1964, pages 79--84. It was submitted on 3 Jul 1963. Translation performed by Sp/7 Charles T. Ostertag, Jr.

At the present time the use of ozone for treating water is drawing all the more attention of hygienists and engineers, since ozonization makes it possible to obtain water which is faultless in an organoleptic respect and safe in an epidemiological respect (Kozhinov, Kulskiy, Gabovich, Vakhler, Taylor, Stumm, Guinvarch, Mescery, Sheller, etc.). These advantages of ozonization over other methods of treating water make it particularly valuable in connection with the greater utilization of open reservoirs for a centralized water supply.

Together with this, the problems of a sanitary-laboratory control of the reliability of disinfection of water by ozone have still not been worked out sufficiently. In particular it is necessary to check the acceptability of the methods, procedures and media which are used for the isolation from the water of E. coli as a sanitary-significative organism, and to substantiate the value of the coli-index 3 as the index of reliability of the disinfection of water from bacteria of the typhoid-paratyphoid-dysentery group, etc.

It is possible to approach the study of these problems following an explanation of those changes which take place in bacteria following the ozonization of water. In the literature available to us we have not encountered any information on the stated problems.

The following method of investigation was used for studying the changes in E. coli under the effect of ozone. To sterile distilled water we added an emulsion of an 18--20 hour agar culture of the typical E. coli with a calculation of 100,000 microbial cells per 1 liter of water. Then the water was ozonized in an experimental apparatus designed by us and following filtration through membrane filters was inoculated on Endo medium. The seedings were incubated (37°) for 8--14 days (sometimes up to 17 days). A calculation of the colonies grown and the nature of growth were noted daily. Ozonized water was also seeded directly on

glucose-peptone medium (Eichman medium) and incubated at 37 and 43°. After 24 and 48 hours seedings were made on Endo medium and rosolic differential agar.

In the incubated cultures we studied the morphology of the bacteria, their ability to take the Gram stain, motility, ability to ferment glucose, lactose, maltose, mannitol, saccharose, and also milk, the formation of indole and hydrogen sulfide, growth in a secondary fermentative probe at 37 and 43°, growth on rosolic agar, and also the presence of catalase and dehydrase.

The investigations showed that following the ozonization of water, E. coli may undergo significant changes.

Following the treatment of water with comparatively small doses of ozone (o.2--0.4 mg per liter) on membrane filters on the Endo medium, by the end of the first day colonies appeared which were shallow and of average dimensions, flat, with even edges, light-red or red, with a lusterless surface. On the second day they increased in size and became dark-red, but did not have the typical metallic luster. Growth was also noted of colonies in the R-form (large, flat colonies with undulating broken edges). In one case there was noted the formation of a colony with a radially streaked spindle. In 2--3 days in a number of cases light-yellow spots appeared on the colonies. These covered almost their entire surface and disappeared in 1--2 days. The colonies fell behind in growth in comparison with colonies which grew from cultures isolated from the water prior to ozonization (see picture, filter to the left). The cultures obtained from atypical colonies we conditionally designated as weakly changed variants.

With an increase in the dose of ozone the number of weakly changed colonies isolated from the water decreased rapidly and sharply changed colonies of E. coli began to appear. In a number of cases from one and the same portion of water these and other bacteria were isolated (see picture, filter in the center). The colonies of sharply changed variants began to appear on the filters by the end of the first day of incubation, however, they were still so fine that it was practically impossible to take them into consideration. The main number of such colonies appeared on the second day and increased somewhat on the third day.

The appearance of new colonies in later periods was not noted.

The dynamics of formation of the stated colonies are presented in the table. At first these were very fine, convex, transparent, colorless colonies. On the 2--3rd day the colonies on Endo medium began to turn rose-colored, without hardly increasing in size. The color change began with the center of the colony, while the edge of the colony still remained

colorless for some time. On the 4--5th day the colonies became red, lustrous, smooth, sharply convex, and reached 2--3 mm in diameter. Gradually they acquired a dark-red color, but they increased in size extremely slowly (see picture, filter on the right). Thus, in the cells of bacteria there apparently took place, as a result of the effect of ozone, a disruption of metabolic processes, which led to a slowing down of their growth and multiplication.

In smears from colonies of the variants a polymorphism of microbes was noted -- from thin elongated bacilli to coccoid bacteria. Following application of the Gram stain they were a pale rose color. A lowering of fuchsin adsorption by the cell indicated the deep change in the protoplasm of the ozonized cells. When taken by a loop the colony was split and emulsified with difficulty. The motility of the variants was reduced, especially significantly in the sharply changed strains, and sometimes it was lacking.

During the seeding of ozonized water on glucose-peptone medium and subsequent incubation at 43°, a slowing down of growth was noted, and in some cases a lack of growth, while in parallel tests incubated at 37°, in the majority of cases growth was noted. It must be noted that turbidity of the seedings began with the upper part of the liquid at the same time that the lower part remained transparent. As a rule, on the surface a delicate colorless film was formed.

Following the seeding from the initial fermentative sample (after 24 hours) following a 24-hour growth on Endo medium, there appeared minute, circular, convex, colorless, lustrous colonies, which grew slowly and gradually (on the third or fourth day) became rose colored. The appearance of such colonies was also noted on the second day of growth.

During the repeated seeding from glucose-peptone medium following 48 hours of growth at 37 and 43°, it was possible to isolate E. coli from a large number of samples.

During the reseeding from Endo medium onto the secondary fermentative sample and incubation at 37°, turbidity of the medium was noted along with the formation of a delicate film and, as a rule, the absence of gas formation, while during incubation of a parallel sample at 43° changes of the medium set in in a smaller number of cases, and were less expressed or were lacking.

When carrying out the investigations by the method of membrane filters, during which a necessary feature of E. coli as a sanitary-significative microorganism is gas formation in the secondary fermentative test at 43°, it was established that during ozonization with large doses E. coli still preserved the capability for gas formation both at 37° and

43° (in the latter case this feature was not always noted). With an increase of the dose of ozone, gas formation was absent on glucose-peptone medium mainly at 43°. A further increase of the dose of ozone led to the fact that an unique feature of growth of E. coli in the secondary fermentative test was the turbidity of the medium, which was less expressed during incubation at 43°. During the treating of water with subbactericidal doses, the E. coli colonies which were grown on membrane filters did not cause changes in the secondary fermentative test either at 37 or at 43° (the so called sharply changed variants).

In connection with this we modified the method of investigation; from the membrane filter we made seedings on rosolic differential medium which, as is known, contains two carbohydrates (at the same time a seeding was made on glucose-peptone medium which was maintained at 43°). The growth of bacteria of E. coli which were changed under the effect of ozone was noted most often on the rosolic medium in the form of dirty-gray colonies. The medium itself became dark-red and gas formation was absent. At the same time in the seedings on glucose-peptone medium changes were lacking (the water was ozonized with subbactericidal doses).

Finally, when carrying out the investigations by the two phase fermentative method with the use of the rosolic medium, an unfavorable effect was also noted in regard to the higher temperature (43°) of incubation of the initial fermentative test. During the reseeding from one and the same test of ozonized water, in some of the test tubes the medium became yellow (formation of an acid), and in the others --dark-red (formation of alkali). As a rule, gas formation was absent. On the beveled part of the medium dirty-gray colonies of E. coli grew, sometimes with a green shade.

A study of the biochemical properties showed that under the influence of ozone, E. coli mainly loses the ability to break down sugar with the formation of a gas. The variants which were isolated from ozonized water formed a cloud and delicate colorless film in meat-peptone broth. When treated with small doses of ozone, indole formation came to an end and the formation of hydrogen sulfide was preserved longer. A further changing of saccharolytic properties led to the formation of lactose defective strains of E. coli. Biochemically inactive variants were isolated from water treated with subbactericidal doses of ozone. Following the seeding of several strains on media with carbohydrates an alkalization of the medium was noted. The change of the medium in this case also began with the upper part of the style. Following the seeding of these same strains on milk an alkalization of the medium was noted.

Thus, as a result of the effect of ozone, E. coli variants appeared which, based on their properties (indifference to carbohydrates or their oxydation, alkalization of milk, absence of indole formation), called to

mind the alkali forming bacteria of the intestinal group, described by Rashba, Stoybun et al., and others.

The same regularity in the variation of E. coli was noted in the ozonization of Dnepr water under laboratory conditions and at the Chasov-Yarskaya Water Works, where the water from the Severnyy Donets--Donbass Canal is ozonized. The difference was that these changes appeared following treatment with large doses of ozone, of which considerably more is required for the disinfection of natural water.

The dehydrogenase activity of E. coli was studied by the method of Tunberg in the Karpenko modification in respect to 8 carbohydrates (rhamnose, saccharose, lactose, glucose, maltose, arabinose, fructose, galactose), 4 alcohols (mannitol, dulcite, sorbitol, inositol), and 10 carbonic acids (malonic, fumaric, succinic, acetic, oxalic, alpha-keto-glutaric, aconitic, glycolid, citric, malic). The dehydrogenase activity was determined based on the duration of discoloration of methylene blue during the dehydrogenation of the substrate by the cells of E. coli at 37°.

In the initial cultures the E. coli dehydrogenated substrates containing carbohydrates and alcohols (except inositol) comparatively rapidly (within the limits of 35--560 minutes), and the majority of carbonic acids considerably slower (an exception was fumaric acid which the bacilli dehydrogenated in the course of 155 minutes). Dehydration of inositol, malonic, acetic, citric and malic acids was absent.

Following the ozonization of water with small doses, in isolated variants of E. coli the dehydrogenation time was increased by 2--3 times for glucose, fructose, galactose, maltose and mannitol, and by 4--6 times for lactose, arabinose, rhamnose and sorbitol. In respect to the majority of carbonic acids, the isolated variants did not possess a dehydrogenase activity (partial dehydrogenation took place in respect to fumaric and malic acids). In contrast to all the remaining substrates the dehydrogenation of saccharose took place 3.2 times more rapidly than in the initial strains.

With a further increase in the dose of ozone the dehydrogenase activity for rhamnose, arabinose, maltose, lactose, mannitol, and sorbitol was lowered all the more. At the same time, for glucose, fructose, galactose and dulcite the dehydrogenase activity was not lowered any further. The time for the dehydrogenation of saccharose was decreased all the more -- by 5.5 times in comparison with the initial strain. The increase of the dehydrogenase activity for saccharose by 2.3--5.5 times apparently should be viewed as an adaptive intensification of the corresponding enzyme production by the cell, which may be related to the group of induced enzymes (Gubarev, Dixon, Webb). During the treatment of water with subbactericidal doses of ozone, in isolated variants the dehydrogenase activity was lowered by 100 times. Thus, the dehydrogenation time

for glucose was increased by 228 times. A lowering of dehydrogenase activity in E. coli was also noted by Trakhtman, Lipinska and Pershin following the effect of another disinfecting agent -- chloride and its compounds. In this manner, ozonization of water caused a depression of the dehydrogenase activity of E. coli in respect to carbohydrates (except saccharose), alcohols and carbonic acids, which in its turn apparently led to a disruption of redox processes which lie at the foundation of metabolism of the microbial cell. This resulted in disruption of growth processes and bacterial multiplication.

A study of catalase activity showed that nonozonized cells immediately broke down hydrogen peroxide. Following ozonization only the weakly changed variants caused the slow formation of very fine vacuoles of oxygen. Following the treatment of water with subbactericidal doses, the variants isolated from the water did not possess a catalase activity.

Conclusions

1. As a result of the effect of ozone, E. coli underwent significant morphological, cultural and biochemical changes.

2. Ozonized bacteria lost the capacity to form gas; lactose deficient as well as biochemically inactive variants appeared. On media with carbohydrates, individual cultures produced alkaline products.

3. For determining the coli-titer of ozonized water an attempt was made to modify the method of membrane filters, in which the secondary fermentation test was replaced by seeding on rosolic differential agar.

4. When determining the coli-titer of ozonized water, the temperature of incubation of the initial fermentation test has an existing significance: Most favorable is a temperature of 37, and not 43°.

5. As a result of the effect of ozone, gas formation was absent in the majority of E. coli variants which developed during incubation of the secondary fermentation sample at 43°.

6. As a result of the ozonization of water a depression was established in the dehydrogenase of E. coli in respect to carbohydrates (except saccharose), alcohols and carbonic acids, which in its turn apparently led to a disruption of the redox processes lying at the base of microbial metabolism. As a result there was noted a disruption of the processes of growth and bacterial multiplication. An increase of dehydrogenase activity for saccharose as a result of the effect of ozone, apparently should be viewed as an adaptive intensification of the corresponding enzyme production by the cell, and it may be regarded as the induced group.

7. Catalase activity in the variants obtained was lowered or lacking altogether. In this manner, in E. coli bacteria under the effect of ozone, the dehydrogenase--oxydase mechanism of respiration was disrupted.

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Colonies of E. coli (seeding by the method of membrane filters, Endo medium, incubation for 10 days at 37°).

Dynamics of the appearance of colonies of E. coli on Endo medium following the seeding of ozonized water by the method of membrane filters

Day of growth	Number of colonies following the inoculation of samples						
	1st	2nd	3rd	4th	5th	6th	7th
1st	4	1	0	0	0	0	0
2nd	39	21	26	7	3	3	1
3rd	39	21	28	7	3	4	1
5th	39	21	28	7	3	4	1
14th	39	21	28	7	3	4	1